

# Identification of the polypeptides encoded in the unassigned reading frames 2, 4, 4L, and 5 of human mitochondrial DNA

(HeLa cells/synthetic peptides/antibodies/mitochondrial translation products)

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Contributed by Giuseppe Attardi, October 21, 1985

**ABSTRACT** In previous work, antibodies prepared against chemically synthesized peptides predicted from the DNA sequence were used to identify the polypeptides encoded in three of the eight unassigned reading frames (URFs) of human mitochondrial DNA (mtDNA). In the present study, this approach has been extended to other human mtDNA URFs. In particular, antibodies directed against the NH<sub>2</sub>-terminal octapeptide of the putative URF2 product specifically precipitated component 11 of the HeLa cell mitochondrial translation products, the reaction being inhibited by the specific peptide. Similarly, antibodies directed against the COOH-terminal nonapeptide of the putative URF4 product reacted specifically with components 4 and 5, and antibodies against a COOH-terminal heptapeptide of the presumptive URF4L product reacted specifically with component 26. Antibodies against the NH<sub>2</sub>-terminal heptapeptide of the putative product of URF5 reacted with component 1, but only to a marginal extent; however, the results of a trypsin fingerprinting analysis of component 1 point strongly to this component as being the authentic product of URF5. The polypeptide assignments to the mtDNA URFs analyzed here are supported by the relative electrophoretic mobilities of proteins 11, 4–5, 26, and 1, which are those expected for the molecular weights predicted from the DNA sequence for the products of URF2, URF4, URF4L, and URF5, respectively. With the present assignment, seven of the eight human mtDNA URFs have been shown to be expressed in HeLa cells.

Mammalian mitochondrial DNAs (mtDNAs) contain, besides five genes of identifiable function, eight unassigned reading frames (URFs), which have a high degree of sequence homology and strong conservation in size and mapping position in different species (1–5). A set of homologous reading frames was subsequently found in the mtDNAs of *Xenopus laevis* (6) and *Drosophila* species (7–9). Surprisingly, these reading frames, with the exception of one (10, 11), have no obvious amino acid sequence homology to any of the reading frames of the mtDNAs from yeasts (12, 13). However, URFs with a partial homology to several of the animal mtDNA URFs occur in other lower eukaryotic organisms—i.e., *Aspergillus nidulans* (14), *Neurospora crassa* (15–17), protozoa (18, 19), unicellular algae (20), and plants (14). The evolutionary persistence of these URFs, as well as the evidence of their transcription into typical mRNAs in HeLa cells (21), suggested very early that they code for functional proteins. Direct evidence that several of these URFs are indeed expressed was first obtained in HeLa cells by the use of antibodies directed against synthetic peptides predicted from the DNA sequence. Thus, the polypeptides

encoded in URF1, URF3, and URF4L were identified on gels, and the identification was confirmed, in the case of URF4L, by analysis of protease fingerprints (22, 23). An independent identification of the polypeptide specified by URF3 was obtained by the analysis of a polymorphism of the gene and of the encoded protein (24). In the work reported here, four additional URFs—i.e., URF2, URF4, URF4L, and URF5—have been shown by the use of anti-peptide antibodies and by fingerprinting analysis to be expressed in HeLa cells, and the corresponding polypeptides have been identified among the mitochondrial translation products.

## MATERIALS AND METHODS

**Peptide Synthesis, Attachment to Carrier Proteins, and Preparation of Antisera.** The techniques used for the synthesis and characterization of the peptides, their attachment to bovine serum albumin, and the preparation and testing of antisera have been described (22, 25). Antibodies directed against the NH<sub>2</sub>-terminal octapeptide of URF2 (anti-URF2-N), against the NH<sub>2</sub>-terminal heptapeptide of URF5 (anti-URF5-N), against the NH<sub>2</sub>-terminal decapeptide of cytochrome *c* oxidase subunit II (anti-COII-N), against the COOH-terminal nonapeptide of URF4 (anti-URF4-C), and against the COOH-terminal heptapeptide of URF4L (anti-URF4L-C) were used.

**In Vivo Labeling of HeLa Cell Mitochondrial Translation Products.** HeLa cells were either labeled directly in methionine-free medium for 2 hr with [<sup>35</sup>S]methionine (1040–1425 Ci/mmol, 10–130 μCi/ml; 1 Ci = 37 GBq) in the presence of emetine (22) at 100 μg/ml or were grown for 22 hr in complete medium in the presence of chloramphenicol (CAP) at 40 μg/ml, then labeled for 2 hr with [<sup>35</sup>S]methionine in methionine-free medium in the presence of cycloheximide at 100 μg/ml, and finally chased for 2 hr in complete medium (26). The above conditions of CAP treatment inhibit mitochondrial protein synthesis in HeLa cells by >90% while allowing growth of the cells at a normal rate for 2 days (27); therefore, they could conceivably allow accumulation in the cytosol and/or mitochondria of cytoplasmic subunits of the enzyme complexes of the inner mitochondrial membrane, as has been reported to occur in *S. cerevisiae* (28) and *N. crassa* (29), and thus increase the stability and/or integration of the mitochondrially synthesized subunits. A marked increase in the 2-hr pulse-labeling of several mitochondrial translation

Abbreviations: URFs, unassigned reading frames; mtDNA, mitochondrial DNA; URF1–URF6, specific URFs in mtDNA; CAP, chloramphenicol; anti-URF5-N and anti-URF2-N, antibodies against the NH<sub>2</sub>-terminal heptapeptide of URF5 and octapeptide of URF2; anti-COII-N, antibody against the NH<sub>2</sub>-terminal decapeptide of cytochrome *c* oxidase; anti-URF4-C and anti-URF4L-C, antibodies against the COOH-terminal nonapeptide of URF4 and heptapeptide of URF4L.

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Table 1. Synthetic peptides and modes of attachment

Source	Sequence*	Attachment procedure†	Degree of BSA substitution‡
URF2 (amino terminus)	Ile-Asn-Pro-Leu-Ala-Gln-Pro-Val-(Glu)	EDC	21
URF4 (amino terminus)	Met-Leu-Lys-Leu-Ile-Val-Phe-(Glu)	EDC	15
URF4 (carboxyl terminus)	(Lys)-Pro-Asp-Ile-Ile-Thr-Gly-Phe-Ser-Ser	GLUT	18
URF4L (amino terminus)	Met-Phe-Leu-Ile-Tyr-Met-Asn-(Glu)	EDC	12
URF4L (carboxyl terminus)	(Lys)-His-Asn-Leu-Asn-Leu-Leu-Gln-X§	GLUT	24
URF5 (amino terminus)	Met-Thr-Met-His-Thr-Thr-Met-(Ala-Glu)	EDC	16
URF5 (carboxyl terminus)	(Lys)-Thr-Leu-Leu-Leu-Ile-Thr	GLUT	32

\*Residues in parentheses were incorporated for attachment purposes. ●, radioactive residue.

†EDC, water-soluble carbodiimide; GLUT, glutaraldehyde.

‡Number of peptide molecules per bovine serum albumin (BSA) molecule.

§X =  $\alpha$ -aminobutyric acid, replacing the encoded COOH-terminal cysteine.

products, including component 11, after CAP-pretreatment has been recently observed (30).

**Immunoprecipitation of Mitochondrial Translation Products and Analysis of Immunoprecipitate.** The mitochondrial fraction was isolated as described (22). The immunoprecipitation experiments were carried out by a modification of the procedure as reported (22). All operations were performed at room temperature unless otherwise specified. In brief, samples of mitochondrial suspension (not sonicated) (110–760  $\mu$ g of protein) were heated at 37°C for 60 min and then at 100°C for 10 min in the presence of 1% NaDodSO<sub>4</sub>. The samples were placed in NET buffer (0.05 M Tris-HCl, pH 8.9 (25°C)/0.15 M NaCl/0.005 M EDTA, final concentrations) containing 0.1% NaDodSO<sub>4</sub>, 1 mg of ovalbumin per ml, 0.001 M phenylmethylsulfonyl fluoride, 0.02% NaN<sub>3</sub>, and 0.002 M methionine. After a 30-min incubation with 125 or 250  $\mu$ l of formaldehyde-fixed *Staphylococcus aureus* Cowan strain I suspension (Zysorbin, Zymed Laboratories, Burlingame, CA), the mixtures were centrifuged in an Eppendorf Microfuge for 2 min. After addition to each supernatant of 250  $\mu$ g or 500  $\mu$ g of the gamma-globulin fraction from the particular antiserum freed of anti-bovine serum albumin antibodies (22) or of the total gamma globulins from normal serum, the mixtures were incubated overnight at 4°C under shaking. After 30 min of further incubation at room temperature, a 10-fold protein excess of a 0.1% NaDodSO<sub>4</sub> mitochondrial lysate from unlabeled cells and 0.25 or 0.50 ml of Zysorbin were added, and the mixtures were kept for 60 min under shaking and then centrifuged in the Microfuge for 2 min. The pellets were washed three times with NET buffer containing 0.1% NaDodSO<sub>4</sub>, 1 mg of ovalbumin per ml, 0.001 M phenylmethylsulfonyl fluoride, and 0.002 M methionine and one time with 0.01 M Tris-HCl (pH 6.7) and finally were extracted with 60  $\mu$ l of 8 M urea/4% NaDodSO<sub>4</sub>/0.01 M Tris-HCl, pH 7.4/0.1%  $\beta$ -mercaptoethanol for 60 min at 37°C and usually for 10 additional min at 50°C. A portion of the extract was run on a NaDodSO<sub>4</sub>/urea/polyacrylamide gel or a NaDodSO<sub>4</sub> linear-gradient polyacrylamide gel (23) prepared with NaDodSO<sub>4</sub> from MCB (technical grade; MCB Manufacturing Chemists, Cincinnati, OH) or Sigma (L5750,  $\approx$ 70% lauryl sulfate) or Bio-Rad, as specified below.

**Fingerprinting Analysis of Tryptic Digests.** The procedure used was as described (22), except that the thin-layer cellulose plate was exposed for autoradiography rather than for fluorography and then was stained with ninhydrin.

## RESULTS

**Preparation of Antipeptide Antibodies.** Table 1 shows the sequences of the peptides of the putative products of URF2, URF4, URF4L, and URF5, which were chosen for synthesis, their mode of attachment to bovine serum albumin, and the degree of substitution obtained. The preparation of anti-COII-N has been described (22).

**Immunoprecipitation of Mitochondrial Translation Products by Anti-URF2-N, Anti-URF4-C, and Anti-URF4L-C Antibodies.** Immunoprecipitation experiments with NaDodSO<sub>4</sub> mitochondrial lysate from HeLa cells labeled for 2 hr with [<sup>35</sup>S]methionine in the presence of emetine at 100  $\mu$ g/ml and gamma globulins from an anti-URF2-N, anti-URF4-C, or anti-URF4L-C antiserum showed that each type of antibody produced a precipitate exhibiting in a NaDodSO<sub>4</sub>/urea/polyacrylamide gel a single specific band. This band corresponded to a mitochondrial translation product and was absent in the normal serum control. One such experiment is shown in Fig. 1A. A band with the same mobility as that of component 11 of the profile of the HeLa cell mitochondrial translation products (31) is visible in the pattern of the immunoprecipitate obtained with gamma globulins from an anti-URF2-N antiserum. Similarly, the electrophoretic pattern of the immunoprecipitate obtained with gamma globulins from an anti-URF4-C antiserum exhibits a band corresponding in mobility to components 4 and 5, while the pattern of the precipitate formed with anti-URF4L-C gamma globulins shows a band corresponding to component 26. In the pattern of the immunoprecipitate obtained with anti-URF4-C antibodies, there is, in addition to the specific band, some trailing heterogeneous material extending up to the origin; this suggests a tendency of the URF4 product to aggregate. Antibodies against the NH<sub>2</sub>-terminal heptapeptide of the URF4 product or of the URF4L product, though strongly reactive with the corresponding peptide, did not precipitate any mitochondrial translation product.

Fig. 2 shows that each specific peptide (added in the amount of 5  $\mu$ g) competed completely with the corresponding mitochondrial translation product in the precipitation by the anti-peptide antibodies. In this experiment, the mitochondrial lysate had been prepared from cells grown for 22 hr in the presence of CAP, then labeled for 2 hr with [<sup>35</sup>S]methionine in the presence of cycloheximide at 100  $\mu$ g/ml, and finally chased for 2 hr in drug-free medium. Note the presence in the electrophoretic pattern of the mitochondrial translation products of bands of higher molecular weight than component 1 because of the labeling of cytoplasmically synthesized proteins during the chase.

Components 4 and 5 were not resolved in the marker pattern of Figs. 1A and 2, in contrast to what had been observed previously (31). Tests carried out under a variety of conditions showed that the source of NaDodSO<sub>4</sub> affected the pattern of the mitochondrial translation products. In the experiments of Figs. 1A and 2, MCB NaDodSO<sub>4</sub> had been used both for solubilization of the mitochondrial fraction and for electrophoresis. When Sigma NaDodSO<sub>4</sub> instead of MCB NaDodSO<sub>4</sub> was used, a marker pattern was obtained in which components 4 and 5 were resolved over a heterogeneous background (Fig. 1B). Furthermore, both components 4 and 5 and the heterogeneous material appeared to be precipitated by anti-URF4-C antibodies (Fig. 1B). It should also be noted

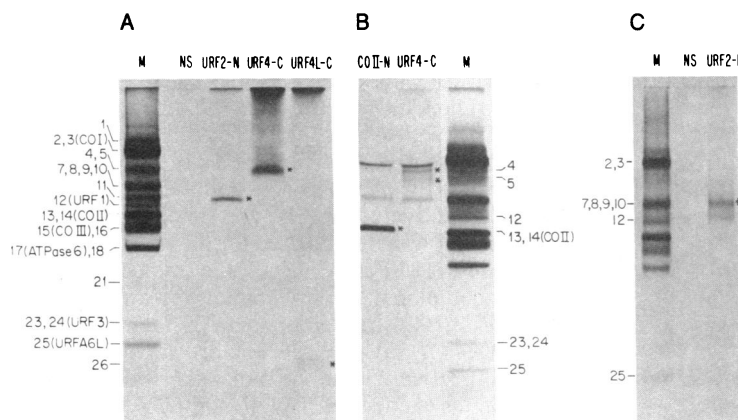


FIG. 1. Fluorograms, after electrophoresis through a NaDodSO<sub>4</sub>/urea/polyacrylamide gel, of HeLa cell mitochondrial translation products precipitated by anti-URF2-N or anti-URF4-C or anti-URF4L-C antibodies. Samples of mitochondrial suspensions from cells labeled for 2 hr with [<sup>35</sup>S]methionine in the presence of emetine at 100 μg/ml (1.2–2 × 10<sup>5</sup> cpm; 75–380 μg of protein) were precipitated with gamma globulins from the indicated antisera, freed of anti-bovine serum albumin antibodies or from normal serum (250–500 μg), and run through a NaDodSO<sub>4</sub>/urea/polyacrylamide gel prepared with MCB NaDodSO<sub>4</sub> (A) or Sigma NaDodSO<sub>4</sub> (B and C). Lanes: NS, control experiment with gamma globulins from a normal rabbit serum; COII-N, immunoprecipitation experiment with gamma globulins from an anti-COII-N antiserum freed of anti-bovine serum albumin antibodies; M, pattern of mitochondrial translation products.

that, when Sigma NaDodSO<sub>4</sub> was used, no well-defined band corresponding to component 11 was visible, in contrast to the case with MCB NaDodSO<sub>4</sub>, and that instead a smear was visible between the two bands corresponding to components 7–10 and 12. Electrophoretic analysis of the immunoprecipitate obtained with anti-URF2-N antibodies in Sigma NaDodSO<sub>4</sub> showed that the precipitated component 11 migrates in this NaDodSO<sub>4</sub> partly as a distinct band moving slightly more slowly than the 7–10 band and partly as faster-moving heterogeneous material extending up to the component 12 band (Fig. 1C). In some electrophoretic runs in Sigma NaDodSO<sub>4</sub> of the total mitochondrial translation products, the discrete band corresponding to component 11 was partly resolved from the 7–10 band (not shown).

Fig. 3 shows the patterns obtained when the mitochondrial translation products precipitated by anti-URF2-N, anti-URF4-C, or anti-URF4L-C antibodies were analyzed in a NaDodSO<sub>4</sub> linear-gradient polyacrylamide slab gel (31) prepared with Sigma NaDodSO<sub>4</sub>. Note that the component precipitated by anti-URF2-N antibodies formed a sharp band at the expected position for component 11 in this gel system (31). In electrophoretic runs of the total mitochondrial translation products in polyacrylamide gradient gels prepared with NaDodSO<sub>4</sub> from different sources (Sigma, MCB, and Bio-Rad), it was found that the band corresponding to compo-

nents 9 and 11 (with component 11 representing a substantial portion of it) had the same relative intensity and mobility with respect to the adjacent bands; these observations suggested that the mobility of component 11 was not affected, as it was in NaDodSO<sub>4</sub>/urea/polyacrylamide gels, by the source of NaDodSO<sub>4</sub>. The component precipitated by anti-URF4-C antibodies yielded, besides a discrete band migrating at the expected position, heterogeneous material trailing over the region of components 2 and 3.

**Immunoprecipitation Experiments with Anti-URF5-N and Anti-URF5-C Antibodies.** Antibodies against the NH<sub>2</sub>-terminal heptapeptide of the putative URF5 product, though very reactive with the corresponding peptide conjugated to ovalbumin, did not react significantly in the immunoprecipitation experiments with the [<sup>35</sup>S]methionine-labeled mitochondrial translation products. However, a very faint band corresponding to component 1, which was apparently absent in the normal serum control, was detectable over the background in the electrophoretic pattern (not shown).

The COOH-terminal hexapeptide of the putative URF5 product, which has a very hydrophobic character, was only sparingly soluble and was difficult to couple to the carrier. This peptide elicited only low-titer antibodies. These were ineffective in precipitating any mitochondrial translation product. Negative results with both the NH<sub>2</sub> terminus- and the COOH terminus-specific antibodies were obtained when they were tested on a Triton X-100 mitochondrial lysate under the conditions used for precipitation of intact complexes (26, 30).

**Fingerprinting Analysis of a Tryptic Digest of Component 1.** The very weak reaction given by the anti-URF5-N antibodies with component 1 of the mitochondrial translation products suggested that this component may be the URF5 product. In agreement with this possibility was the fact that URF5 is the only reading frame large enough to code for a protein of the size of component 1 ( $M_r \approx 51,000$ , as estimated on the basis

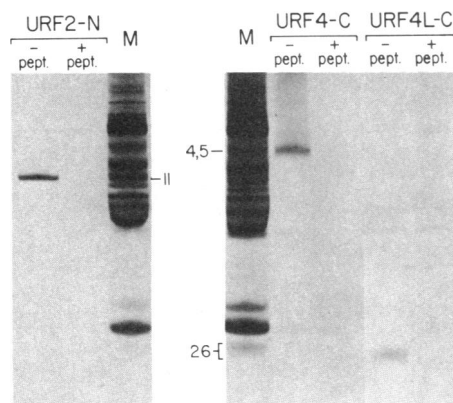


FIG. 2. Specific peptide inhibition of immunoprecipitation of components 11, 4 and 5, and 26, respectively, by anti-URF2-N, anti-URF4-C, and anti-URF4L-C antibodies. Samples of a mitochondrial suspension (2 × 10<sup>5</sup> cpm; 130 μg of protein) from cells grown for 22 hr in the presence of chloramphenicol at 40 μg/ml, then labeled for 2 hr with [<sup>35</sup>S]methionine in methionine-free medium in the presence of cycloheximide at 100 μg/ml, and finally chased for 2 hr in complete medium were precipitated by anti-URF2-N, anti-URF4-C, or anti-URF4L-C gamma globulins (125 μg) in the absence (–) or presence (+) of 5 μg of URF2-N, URF4-C, or URF4L-C peptide (pept.), respectively. Lanes M show the pattern of mitochondrial translation products.

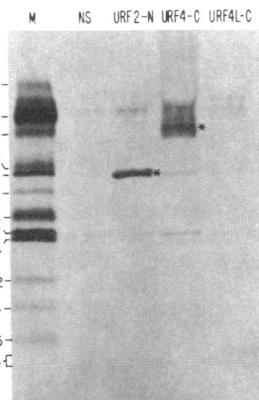


FIG. 3. Fluorogram after electrophoresis through a NaDodSO<sub>4</sub>/polyacrylamide gradient gel (in Sigma NaDodSO<sub>4</sub>), of HeLa cell mitochondrial translation products precipitated by gamma globulins from an anti-URF2-N, anti-URF4-C, or anti-URF4L-C antiserum. The samples were equivalent to those electrophoresed through a NaDodSO<sub>4</sub>/urea/polyacrylamide gel in Fig. 1A. Other lanes: NS, normal serum control; M, pattern of mitochondrial translation products.

of its electrophoretic mobility (31); see below]. In order to verify this tentative assignment, advantage was taken of the fact that this product contains the sequence Lys-Met-Lys, which would yield, upon trypsin digestion, a methionyllysine dipeptide. Therefore, a bidimensional fingerprinting analysis of component 1 after trypsin digestion was carried out.

Fig. 4 shows the fingerprinting pattern of a tryptic digest of component 1, which was run together with a chemically synthesized methionyllysine marker (22). Many spots of different intensities are visible in the autoradiogram. One of them corresponds perfectly in position and shape to the methionyllysine marker. A much less intense spot, which has migrated somewhat less toward the cathode, corresponds in position to a minor portion of the marker representing derivatives of the methionyllysine dipeptide in which the methionine moiety is oxidized to the sulfoxide and sulfone level (as shown by comparison with the migration of the completely oxidized marker run at the border of the plate) (22). The methionyllysine dipeptide is one of the most intense spots in the autoradiogram, in spite of the fact that several tryptic digestion products containing two to four methionine residues, and therefore more highly labeled, would be expected. This is attributable to the fact that the dipeptide is preferentially eluted from the gel during the enzyme digestion.

### DISCUSSION

Component 11 of the HeLa cell mitochondrial translation products, the polypeptide specifically precipitated in this work by anti-URF2-N antibodies, has been estimated previ-

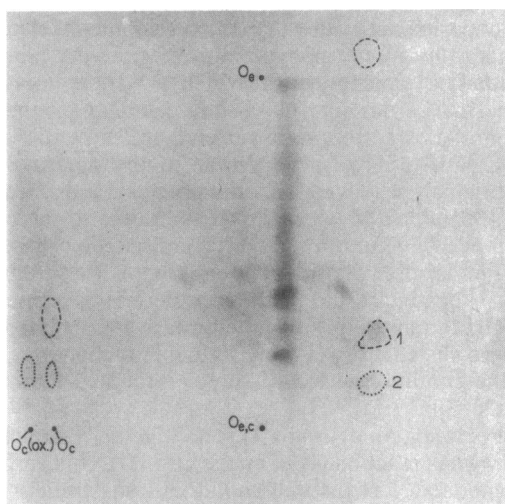


FIG. 4. Autoradiogram of a bidimensional fingerprint of the products of trypsin digestion of component 1. [ $^{35}$ S]Methionine-labeled component 1 was isolated from a NaDodSO<sub>4</sub>/urea/polyacrylamide gel electrophoresis and digested with trypsin as described (22). The lyophilized digest was spotted in the lower half of a TLC plate (origin marked O<sub>e,c</sub>) together with a sample of methionyllysine dipeptide marker; another sample of the marker was spotted in the upper half of the plate (origin marked O<sub>e</sub>). After electrophoresis, the plate was dried, a sample of the marker and another sample of marker that had been completely oxidized with 3% hydrogen peroxide to give 50% sulfoxide derivative and 50% sulfone derivative (22) were spotted in the left half of the plate [origins marked O<sub>e</sub> and O<sub>e</sub>(ox.), respectively], and chromatography was run. The first dimension (electrophoresis) is in the horizontal direction (with the cathode on the right); the second dimension (chromatography) is from bottom to top. The dashed profiles indicate the positions of the native methionyllysine dipeptide, as revealed by ninhydrin staining; the dotted profiles are the positions of the oxidized dipeptide. In spot 2, the stained profile extends beyond the right border of the radioactive profile, presumably because of the presence of a stained peptide derived from self-digestion of trypsin, which overlaps the oxidized peptide (22).

ously to have a  $M_r$  of  $\approx 25,000$  on the basis of its electrophoretic mobility in a NaDodSO<sub>4</sub>/urea/polyacrylamide gel (31). This molecular weight is smaller by  $\approx 36\%$  than that expected for the protein encoded in URF2 ( $M_r$  38,900; ref. 1). Similarly the molecular weight estimates, based on electrophoretic data, for components 4 and 5, which are specifically immunoprecipitated by anti-URF4-C antibodies ( $M_r$ s 39,000 and 36,000, respectively; ref. 31), are smaller by  $\approx 24\text{--}30\%$  than the molecular weight predicted for the product of URF4 ( $M_r$  51,400; ref. 1). Furthermore, the molecular weight estimates for component 26, precipitated by anti-URF4L-C antibodies ( $M_r$  3,500), and component 1, identified as the URF5N product ( $M_r$  51,000; ref. 31), are smaller by  $\approx 67\%$  and  $\approx 23\%$ , respectively, than the molecular weights expected for the proteins encoded in URF4L and URF5 ( $M_r$ s 10,700 and 66,600, respectively; ref. 1). However, a similar bias in size estimates based on electrophoretic mobility in NaDodSO<sub>4</sub>/polyacrylamide gels has previously been observed for other hydrophobic mitochondrial translation products (22, 23, 32, 33).

A plot of electrophoretic mobility vs. molecular weight predicted from the DNA sequence for the mitochondrially synthesized polypeptides so far identified (Fig. 5) clearly shows that all these polypeptides, with the exception of cytochrome *c* oxidase and the URFA6L product, have relative mobilities in a NaDodSO<sub>4</sub>/urea/polyacrylamide gel that are consistent with their expected molecular weights. Therefore, the bias in size estimates based on electrophoretic mobility in NaDodSO<sub>4</sub>/polyacrylamide gels of mtDNA-encoded hydrophobic proteins appears to be due to the use of proteins water-soluble in the absence of detergents for the standard curve. In fact, the latter proteins are expected to bind less NaDodSO<sub>4</sub> than do hydrophobic proteins of equivalent molecular weight and to migrate more slowly in an electric field. Cytochrome *c* oxidase and the URFA6L product exhibit a lower-than-predicted mobility, presumably due to a lower degree of NaDodSO<sub>4</sub> binding. In fact, the URFA6L product is unique among the human mitochondrially synthesized polypeptides for having the amino acid composition of a water-soluble protein (34); furthermore, the hydropathy plot of human cytochrome *c* oxidase reveals that the COOH terminus proximal half of the protein is only slightly hydrophobic (30).

The absolute specificity of the precipitation reactions detected here and the observation that the polypeptides precipitated by the specific antibodies have relative electro-

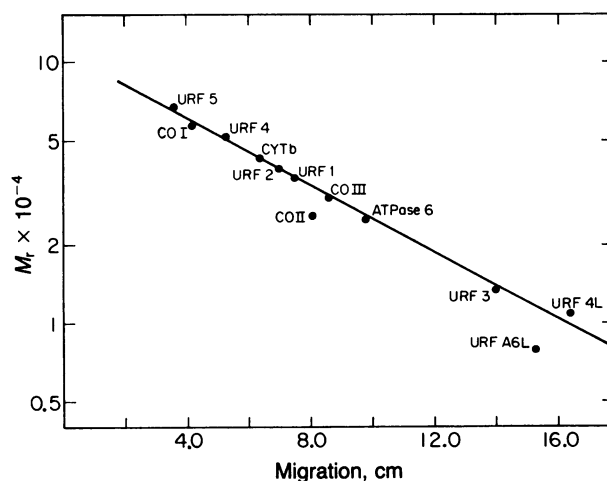


FIG. 5. Plot of electrophoretic mobility of the human mitochondrial translation products in a NaDodSO<sub>4</sub>/urea/polyacrylamide gel (in MCB NaDodSO<sub>4</sub>) versus molecular weight predicted from the DNA sequence.

phoretic mobilities perfectly consistent with the molecular weights predicted from the DNA sequence for the products of the corresponding reading frames strongly support the conclusion that component 11, components 4 and 5, and component 26 are encoded respectively in *URF2*, *URF4*, and *URF4L*. In the case of component 1, which reacted only very weakly with the anti-*URF5*-N antibodies, the tentative identification as the *URF5* product, as also suggested by size considerations, was corroborated by fingerprinting analysis of a tryptic digest of this component—in particular by the detection of the dipeptide methionyllysine in this digest. Of the three amino acid sequences that would result in the formation of the dipeptide methionyllysine after trypsin digestion, the sequence Arg-Met-Lys and the NH<sub>2</sub>-terminal Met-Lys sequence do not occur in any expected mitochondrial gene product; on the other hand, the sequence Lys-Met-Lys is found in two other gene products besides the *URF5* product—i.e., in the polypeptides encoded by *URF2* and *URFA6L* (1). However, the much smaller size of the products of these two reading frames and the lack of reactivity of anti-*URF2*-N (this work) and anti-*URFA6L* antibodies (22) with component 1 clearly exclude *URF2* and *URFA6L* as the genes encoding component 1.

Components 4 and 5, which are partially resolved under appropriate conditions of electrophoresis, were both precipitated by anti-*URF4*-C; therefore, presumably they are both encoded by *URF4*. The nature of the change of the *URF4* product, which results in two components with distinct electrophoretic mobilities, is not known, nor has it been investigated further.

The striking effect that the source of NaDodSO<sub>4</sub> can have on the electrophoretic mobility of some proteins has been previously reported (35, 36). In fact, in a NaDodSO<sub>4</sub> discontinuous buffer system, both the separation of the proteins (35, 36) and their order of electrophoretic migration (35) are influenced by the source of NaDodSO<sub>4</sub>. This phenomenon is presumably related to the presence in the NaDodSO<sub>4</sub> from different sources of various amounts of contaminating alkyl chains longer than C<sub>12</sub>, with higher affinities for certain sequences. However, the presence of urea also can play a role in the phenomenon (36; this work).

With the present identification of the polypeptides encoded in *URF2*, *URF4*, *URF4L*, and *URF5*, seven of the eight human URFs have been shown to be expressed in HeLa cells. Of these seven URFs, *URFA6L*, because of its homology to the yeast mtDNA gene (*aap1*) encoding subunit 8 of the ATP synthase complex (10, 11) and of the results of immunoprecipitation experiments (37), appears to code for a component equivalent to ATPase 8. The other six URFs—i.e., *URF1*, *URF2*, *URF3*, *URF4*, *URF4L*, and *URF5*—have been shown recently to encode subunits of the respiratory chain NADH dehydrogenase, and it has been proposed that their designations be changed to ND [for NADH dehydrogenase (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, and *ND5*); ref. 26].

The technical assistance of Ms. Doris Finch and Ms. Arger Drew is gratefully acknowledged. These investigations were supported by National Institutes of Health Grants GM-11726 (to G.A.) and National Science Foundation grant PCM-8118172 to R.F.D.

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